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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
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EXAMINER	
ANGELL, JON E	

ART UNIT	PAPER NUMBER
1635	

DATE MAILED: 10/10/2006

Please find below and/or attached an Office communication concerning this application or proceeding.

Office Action Summary	Application No.	Applicant(s)	
	10/080,797	BRAZZELL ET AL.	
	Examiner	Art Unit	
	Jon Eric Angell	1635	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *05 May 2006 and 02 June 2006*.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1-3,8,27-33,38-41,43 and 45-50* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *1-3,8,27-33,38-41,43 and 45-50* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

Continued Examination Under 37 CFR 1.114

A request for continued examination under 37 CFR 1.114, including the fee set forth in 37 CFR 1.17(e), was filed in this application after final rejection. Since this application is eligible for continued examination under 37 CFR 1.114, and the fee set forth in 37 CFR 1.17(e) has been timely paid, the finality of the previous Office action has been withdrawn pursuant to 37 CFR 1.114. Applicant's submission filed on 5/5/2006 and 6/2/2006 have been entered.

1. Applicant's arguments are addressed on a per section basis. The text of those sections of Title 35, U.S. Code not included in this Action can be found in a prior Office Action. Any rejections not reiterated in this action have been withdrawn as being obviated by the amendment of the claims and/or applicant's arguments.

Claims 1-3, 8, 27-33, 38-41, 43, 45-50 are currently pending and are examined herein.

Claim Rejections - 35 USC § 102

1. The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

2. Claims 1-3, 8, 27, 28, 30, 31, 43, and 45-47 are rejected under 35 U.S.C. 102(b) as being anticipated by Leboulch et al. (WO 99/26480, cited as IDS reference AN), for the reasons of record (e.g., see the Action mailed on 3/14/05), reiterated below for convenience.

As previously indicated, Leboulch teaches a method for treating a human patient suffering from diabetic retinopathy (which can result in corneal, retinal and iris neovascularization) by administering to said patient a nucleic acid molecule which expresses endostatin wherein expression of the endostatin polypeptide in the patient inhibits angiogenesis in the vicinity of the retina (e.g., see claim 33, page 33-34). Specifically, Leboulch teaches that the gene therapy vector can be a retroviral vector, adenoviral vector or adenoviral-associated vector (AAV) (see p. 5, lines 19-21). Furthermore, Leboulch indicates that the gene therapy vector can be administered by any method that allows the vector to reach the target cells, such as injection to the target tissue, wherein the target tissue can be the retina of the eye (e.g., see p. 11, lines 10-22; and p. 14, lines 2-14; and claim 33). Therefore, Leboulch anticipates the instant claims.

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

This application currently names joint inventors. In considering patentability of the claims under 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later

invention was made in order for the examiner to consider the applicability of 35 U.S.C. 103(c) and potential 35 U.S.C. 102(e), (f) or (g) prior art under 35 U.S.C. 103(a).

Claims 1 and 29 are rejected under 35 U.S.C. 103(a) as being unpatentable over Leboulch et al. (WO 99/26480, cited as IDS reference AN) in view of Keshet et al. (Journal of Clinical Investigation, 1999) and further in view of Otani et al. (Investigative Ophthalmology & Visual Science, 1999) for the reasons of record (e.g., see the Action mailed on 3/14/05), reiterated below for convenience.

As previously indicated, Leboulch teaches a method for treating a human patient suffering from diabetic retinopathy (which can result in corneal, retinal and iris neovascularization) by administering to said patient a nucleic acid molecule which expresses endostatin wherein expression of the endostatin polypeptide in the patient inhibits angiogenesis in the vicinity of the retina (e.g., see claim 33, page 33-34). Specifically, Leboulch teaches that the gene therapy vector can be a retroviral vector, adenoviral vector or adenoviral-associated vector (AAV) (see p. 5, lines 19-21). Furthermore, Leboulch indicates that the gene therapy vector can be administered by any method that allows the vector to reach the target cells, such as injection to the target tissue, wherein the target tissue can be the retina of the eye (e.g., see p. 11, lines 10-22; and p. 14, lines 2-14; and claim 33).

Leboulch does not teach that the method can be used to treat choroidal neovascularization.

Keshet et al. teaches that endostatin is an antiangiogenic peptide that inhibits VEGF activity. Specifically, Keshet et al. teaches, "Endostatin was shown to inhibit VEGF-induced

endothelial cell migration in vitro and to have anti-tumor activity in vivo, without any apparent signs of toxicity." (See p. 1500, 1st column, lines 3-6).

Furthermore, it was recognized in the art that vascular endothelial growth factor (VEGF) is involved in choroidal neovascularization (CN). For instance, Otani et al. teaches,

> "Recent histological and immunohistochemical studies of experimentally produced and surgically excised CNVMs [choroidal neovascular membranes] have indicated that VEGF, transforming growth factor beta (TGFβ), acidic fibroblast growth factor (aFGF) and basic fibroblast growth factor (bFGF) are involved in the mechanism of CNVM formation associated with ARMD [age-related macular degeneration]. Because VEGF has great selectively for endothelial cells, it is considered to be a critical angiogenic factor in the development of CVMN, even though the mechanism of CNVM is not fully understood." (Emphasis added; see paragraph bridging pages 1912-1913).

It is also noted that Otani et al. teaches, "Present findings that Ang2 and VEGF are co-upregulated and that Tie2 is expressed in a variety of cell types in CVNMs further support a crucial role of the interaction between VEGF and Ang2 in pathologic angiogenesis of CNVM formation." (See p. 1912, Abstract).

Therefore, it would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to use the method taught by Leboulch to ameliorate or reduce the rate of choroidal neovascularization in a subject with a reasonable expectation of success.

Since the teachings of the prior art indicate that (1) Endostatin is an antiangiogenic factor that inhibits VEGF activity, (2) Endostatin can be used in gene therapy methods to inhibit neovascularization, and (3) VEGF is known to be involved in choroidal neovascularization (e.g., see Otani et al.) one of ordinary skill in the art would have been motivated to use the method of Leboulch to inhibit choroidal neovascularization.

Claims 1 and 32 are rejected under 35 U.S.C. 103(a) as being unpatentable over Leboulch et al. (WO 99/26480, cited as IDS reference AN) in view of US Patent 6,106,826 (Brandt et al.) for the reasons of record (e.g., see the Action mailed on 3/14/05), reiterated below for convenience.

As previously indicated, Leboulch teaches a method for treating a human patient suffering from diabetic retinopathy (which can result in corneal, retinal and iris neovascularization) by administering to said patient a nucleic acid molecule which expresses endostatin wherein expression of the endostatin polypeptide in the patient inhibits angiogenesis in the vicinity of the retina (e.g., see claim 33, page 33-34). Specifically, Leboulch teaches that the gene therapy vector can be a retroviral vector, adenoviral vector or adenoviral-associated vector (AAV) (see p. 5, lines 19-21). Furthermore, Leboulch indicates that the gene therapy vector can be administered by any method that allows the vector to reach the target cells, such as injection to the target tissue, wherein the target tissue can be the retina of the eye (e.g., see p. 11, lines 10-22; and p. 14, lines 2-14; and claim 33).

Leboulch does not teach that vector is administered intravitreally.

Brandt teaches gene therapy vectors which can be used to deliver therapeutic genes for gene therapy, and specifically teaches an HSV vector as well as an adenoviral vector and adeno-associated vector for use in gene therapy of the eye wherein the vector can be delivered to the eye by intravitreally injecting the vector as well as subretinally and intraocullarly delivering the vector, for therapeutic purposes, such as macular degeneration. (e.g., see abstract, column 5, lines 5-20, column 8,lines 57-65, and column 9 lines 15-20).

Therefore, it would have been prima facie obvious to one of ordinary skill in the art at the time of filing to modify the method taught by Leboulch such that the vector used was delivered intravitreally with a reasonable expectation of success.

The motivation to modify the method of Leboulch is supplied by Brandt who specifically teaches that intravitreal delivery of a therapeutic vector is an effective administration for gene therapy of eye diseases.

Claims 1, 33 and 38 are rejected under 35 U.S.C. 103(a) as being unpatentable over Leboulch et al. (WO 99/26480, cited as IDS reference AN) in view of US Patent 6,555,107 (Poeschla et al.) for the reasons of record (e.g., see the Action mailed on 3/14/05), reiterated below for convenience.

As previously indicated, Leboulch teaches a method for treating a human patient suffering from diabetic retinopathy (which can result in corneal, retinal and iris neovascularization) by administering to said patient a nucleic acid molecule which expresses endostatin wherein expression of the endostatin polypeptide in the patient inhibits angiogenesis in the vicinity of the retina (e.g., see claim 33, page 33-34). Specifically, Leboulch teaches that the gene therapy vector can be a retroviral vector, adenoviral vector or adenoviral-associated vector (AAV) (see p. 5, lines 19-21). Furthermore, Leboulch indicates that the gene therapy vector can be administered by any method that allows the vector to reach the target cells, such as injection to the target tissue, wherein the target tissue can be the retina of the eye (e.g., see p. 11, lines 10-22; and p. 14, lines 2-14; and claim 33).

Leboulch does not teach that vector is a lentiviral vector or that that the vector is a bovine immunodeficiency viral vector.

Poeschla teaches methods of gene therapy for the eye wherein a lentiviral vector, specifically a bovine immunodeficiency vector, is used to deliver and express the therapeutic gene in the eye of the subject. (For example, see abstract, column 2, lines 10-35; column 4, lines 34-41; column 11, 45-55).

Therefore, it would have been prima facie obvious to one of ordinary skill in the art at the time of filing to modify the teaching of Leboulch such that the gene therapy vector used is the bovine immunodeficiency viral vector taught by Poeschla (which is a lentiviral vector) with a reasonable expectation of success.

The motivation to make such a modification is provided by Poeschla. Poeschla teaches that the BIV vector is better for transfecting difficult to target non-dividing cells of the nervous system including eye cells.

Claims 1, 33, 38-41 and 48-50 are rejected under 35 U.S.C. 103(a) as being unpatentable over Leboulch et al. (WO 99/26480, cited as IDS reference AN) in view of US Patent 6,555,107 (Poeschla et al.) and further in view of US Patent 6,106,826 (Brandt et al.) for the reasons of record (e.g., see the Action mailed on 3/14/05), reiterated below for convenience.

As previously indicated, Leboulch teaches a method for treating a human patient suffering from diabetic retinopathy (which can result in corneal, retinal and iris neovascularization) by administering to said patient a nucleic acid molecule which expresses

endostatin wherein expression of the endostatin polypeptide in the patient inhibits angiogenesis in the vicinity of the retina (e.g., see claim 33, page 33-34). Specifically, Leboulch teaches that the gene therapy vector can be a retroviral vector, adenoviral vector or adenoviral-associated vector (AAV) (see p. 5, lines 19-21). Furthermore, Leboulch indicates that the gene therapy vector can be administered by any method that allows the vector to reach the target cells, such as injection to the target tissue, wherein the target tissue can be the retina of the eye (e.g., see p. 11, lines 10-22; and p. 14, lines 2-14; and claim 33).

Leboulch does not teach that vector is a lentiviral vector such as a bovine immunodeficiency viral (BIV) vector or that the lentiviral/BIV vector is administered intraocularly, subretinally or intravitreally.

Poeschla teaches methods of gene therapy for the eye wherein a lentiviral vector, specifically a bovine immunodeficiency vector, is used to deliver and express the therapeutic gene in the eye of the subject. (For example, see abstract, column 2, lines 10-35; column 4, lines 34-41; column 11, 45-55).

Brandt teaches gene therapy vectors which can be used to deliver therapeutic genes for gene therapy, and specifically teaches an HSV vector as well as an adenoviral vector and adeno-associated vector for use in gene therapy of the eye wherein the vector can be delivered to the eye by intravitreally injecting the vector which would necessarily encompass sub-retinal as well as intraocular delivery (e.g., see abstract, column 5, lines 5-20, column 8, lines 57-65, and column 9 lines 15-20).

Therefore, it would have been prima facie obvious to one of ordinary skill in the art at the time of filing to modify the teaching of Leboulch such that the bovine immunodeficiency viral

vector taught by Poeschla (which is a lentiviral vector) is used to deliver and express the therapeutic gene and to deliver the lentiviral/BIV vector by intravitreally, subretinally or intraocullarly injecting the gene therapy vector with a reasonable expectation of success.

The motivation to make such a modification is provided in part by Brandt who specifically teaches that adenoviral and AAV vectors can be used to treat eye disease by intravitreally, subretinally or intraocullarly delivering the therapeutic vector; and in part by Poeschla who teaches that the BIV vector is better for transfecting difficult to target non-dividing cells of the nervous system including eye cells.

Response to Arguments and Declaration

The Dr. Sheila Connelly Declarations under 37 CFR 1.132 filed 2/13/2006 and 5/5/2006 are insufficient to overcome the rejection of claims based upon 35 U.S.C. 102(b) and 35 U.S.C. 103(a) as set forth in the last Office action for the following reasons.

Dr. Connelly indicates that she has read and understands the Leboulch et al. reference (WO 99/26480), and it is her opinion that at the time of filing (February 2001) although endostatin was demonstrated by Dr. Judah Folkman's laboratory in the mid 1990's to be an anti-angiogenic agent that could treat some cancers in mice, "[E]ndostatin rapidly fell out of favor with scientists as they were unable to repeat the studies from Dr. Folkman's laboratory." Dr. Connelly cites a Wall Street Journal article from 1998 entitled "Novel cancer approach stumbles as others fail to repeat success". Dr. Connelly states, "The prevailing attitude at the time was significant skepticism about the therapeutic utility of endostatin." Dr. Connelly asserts that the scientific literature is replete with articles of endostatin failure and cites several articles including

Jouanneau et al. 2001, Eisterer et al. 2002, and Bachelot et al. 2002. Dr. Connelly correctly identifies Dr. Leboulch, an inventor of the WO 97/26480 application, as co-author of the Jouanneau et al., Eisterer et al., and Bachelot et al. references and asserts that Dr. Leboulch was "on the forefront of researchers discrediting endostatin." In her second Declaration Dr. Connelly states, "The prevailing attitude at the time of Leboulch et al. was significant skepticism about the therapeutic utility of endostatin." Dr. Connelly asserts that Leboulch et al. "recognize the unpredictability of having endostatin function for treating cancer, and then provide a generic listing of tissues without any discussion of a particular vector or target cell type, etc." Dr. Connelly also indicates that she was advised that many of the Examples were not actually conducted which leads her to conclude that Leboulch does not provide any information that endostatin is antiangiogenic in the eye.

In assessing the weight to be given expert testimony, the examiner may properly consider, among other things, (1) the nature of the fact sought to be established, (2) the strength of any opposing evidence, (3) the interest of the expert in the outcome of the case, and (4) the presence or absence of factual support for the expert's opinion.

(1) In the instant case, the nature of the fact sought to be established is whether or not, at the time the instant application was filed, Leboulch et al. (WO 99/26480) provides an enabling disclosure for ameliorating or reducing the rate of ocular neovascularization in an individual afflicted with ocular neovascularization by administering to the eye or eyes of the individual a viral vector that operably encodes and expresses a functionally active endostatin. It is not required that Leboulch et al. provide an enabling disclosure for using endostatin gene therapy to treat cancer. It is noted that, at the time of filing, (1) endostatin was recognized to be

an anti-angiogenic factor, (e.g., see U.S. Patent 5,8854,205 and 6,174,861), and (2) one of skill in the art would know that there were methods available to deliver and express a therapeutic gene in an eye (e.g., see US Patents 5,827,702, 6,201,104, 6,106,826, and 6,555,107, all previously cited)

(2) There is significant evidence which opposes the conclusion of the Connelly declarations that Leboulch et al. is not enabled for treating ocular neovascularization of the eye by endostatin gene therapy. For instance, U.S. Patent No. 5,854,205 establishes that endostatin is an antiangiogenic protein, and U.S. Patent No. 6,174,861 claims a method of inhibiting angiogenesis by administering endostatin. Furthermore, one of skill in the art would know that there are methods for delivering and expressing therapeutic genes ion the eye (e.g., see US Patents 5,827,702, 6,201,104, 6,106,826, and 6,555,107, all previously cited). Therefore, one of skill in the art aware of the above indicated prior art, would, without evidence to the contrary, consider the teaching of Leboulch et al. that expressing the endostatin gene in an eye of an individual suffering from ocular neovascularization to be enabled.

(3) It appears that Dr. Connelly may have an interest in the case since she is a co-author of a journal article entitled "Intraocular expression of endostatin reduces VEGF-induced retinal vascular permeability, neovascularization and retinal detachment." (FASEB Journal, published Marh 28, 2003). The FASEB article also includes Dr. Campochiaro, Dr. Kaleko and Dr. Luo as co-authors, all of which are also listed as inventors of the instant application. Furthermore, the FASEB article also indicates that Dr. Connelly, Dr. Kaleko and Dr. Luo are all employed by Advances Vision Therapies, Inc.

(4) The articles cited by Dr. Connelly, which provide the factual support for her position, do not indicate that, at the time of filing, Leboulch et al. is not enabled for treating ocular

neovascularization of the eye by endostatin gene therapy. Although Dr. Connelly asserts that the scientific literature is replete with articles of endostatin failure, none of the articles cited by Dr. Connelly provide evidence that indicates that it is unpredictable whether or not endostatin is an anti-angiogenic factor. All of the references cited by Dr. Connelly indicate, at best, that there is inconsistency in using endostatin as an anti-cancer agent. It is respectfully pointed out that at least some of the references cited by Dr. Connelly recognize endostatin as an anti-angiogenic factor.

For instance, Jouanneau et al. teach, "Here, we have evaluated the efficacy of one of the most promising natural inhibitors of angiogenesis described to date, endostatin, in a human neuroblastoma xenograft model in nude mice... The in vitro activity of soluble endostatin was confirmed on bovine capillary endothelial cells and human umbilical vein endothelial cells." (See abstract). Figure 2 of Jouanneau demostrates the in vitro anti-angiogenic activity of endostatin.

Eisterer et al. teach, "A variety of studies have indicated endostatin to be a potent anti-angiogenic agent both in vitro and in vivo, and a human malignancy that might be sensitive to endostatin is human B-lineage acute lymphoblastic leukemia (B-ALL)." (See abstract) Although Eisterer does not teach that endostatin is an effective anti-cancer agent, there is no doubt that Eisterer recognizes the antiangiogenic activity of endostatin.

Bachelot teaches:

> "One of the most promising of these recently described natural inhibitors of angiogenesis is endostatin, a C-terminal fragment of collagen XVIII. In-vitro, endostatin strongly inhibits endothelial cell proliferation and migration. Initial in-vivo studies were impressive, recombinant endostatin was shown to induce regression and prevent the growth of experimental tumors in mice. Several studies by independent teams were published thereafter; they either described different forms of the recombinant protein, or

> developed gene therapy approaches. Most groups have shown perceptible activity in mouse tumor models, albeit without evidence of tumor regression." (Emphasis added, See abstract).

Therefore, Bachelot teaches that most studies have shown perceptible endostatin antiangiogenic activity in mouse tumor models even if evidence of tumor regression was not seen.

Furthermore, U.S. Patents 5,854,205 and 6,174,861 teach that endostatin is an antiangiogenic molecule which can be used to inhibit angiogenesis in a subject. For instance, 6,174,861 explicitly teaches,

> "It is yet another object of the present invention to provide a method and composition for treating diseases and processes that are mediated by angiogenesis including, but not limited to, hemangioma, solid tumors, leukemia, metastasis, telangiectasia psoriasis scleroderma, pyogenic granuloma, myocardial angiogenesis, plaque neovascularization, coronary collaterals, cerebral collaterals, arteriovenous malformations, ischemic limb angiogenesis, corneal diseases, rubeosis, neovascular glaucoma, diabetic retinopathy, retrolental fibroplasia, arthritis, diabetic neovascularization, macular degeneration, wound healing, peptic ulcer, fractures, keloids, vasculogenesis, hematopoiesis, ovulation, menstruation, and placentation." (Emphasis added, see column 4, lines 10-23).

It is not clear how Dr. Connelly was advised that many of the Examples in Leboulch were not actually conducted. Furthermore, there is no evidence presented which indicates that that endostatin would not function as an antiangiogenic in the eye.

Therefore, given the very strong evidence indicating that endostatin is an antiangiogenic protein and considering that methods of gene therapy of the eye were taught in the prior art, the conclusion is inescapable that Leboulch et al. provides an enabling disclosure for treating ocular neovascularization of the eye by delivering to the eye a gene therapy vector that encodes and expresses endostatin.

Applicants argue that Leboulch (WO 99/26480) is not enabling for ocular gene therapy, as is required, and thus does not anticipate the instant claims. Applicants cite several court cases in support of their position. For instance, Applicants cite MPEP §2121.01, which indicates that the test for determining whether a prior art disclosure is sufficient and effective is whether the reference contains an enabling disclosure (In re Hoeksema, 399 F.2d 269, 158 USPQ 596 (CCPA 1964). Also, In re Donohue, 766 F.2d 531,226 USPQ 619 (Fed. Cir. 1985) which indicates, a reference is considered to contain an enabling disclosure if the public was in possession of the disclosed subject matter before the date of invention. Such possession is effective if the artisan could have combined the description in the publication with her or his own knowledge to make the claimed invention. Applicants also cite In re LeGriece, 301 F.2d 929, 49 CCPA 1124 (1962) as being instructive on that point. Applicants also cite U.S. v. Adams, 383 U.S. 39, 148 USPQ 479 (1965), Process Control Corporation v. HydReclaim Corporation, 190 F.3d 1350, 52 USPQ 2d 1029 (Fed. Cir. 1999), and In re Cook, 439 F.2d 730, 169 USPQ 298 (CCPA 1971).

All of the cited cases have been reviewed, and the Examiner does not take issue with the notion that a prior art reference must be enabled in order to be considered effective. However, for the reasons indicated herein, Applicants have not provided sufficient evidence to demonstrate that Leboulch et al. is not enabled for ameliorating or reducing the rate of ocular neovascularization in an individual afflicted with ocular neovascularization by administering to the eye or eyes of the individual a viral vector that operably encodes and expresses a functionally active endostatin. The evidence provided by Applicants, at best, indicates that endostatin may not always work as an anti-cancer agent. There is no evidence presented which would bring into question endostatin's ability to inhibit angiogenesis in any tissue, let alone the eye.

Furthermore, it is respectfully pointed out that claim 33 of WO 99/26480 explicitly claims using gene therapy to treat diabetic retinopathy wherein endostatin expression inhibits angiogenesis in the vicinity of the retina. Furthermore, page 2 (last full paragraph) of the WO 99/26480 document clearly teaches ex vivo and in vivo methods and identifies in vivo therapy as a preferred embodiment. WO 99/26480 also indicates that methods preferably involve delivery of the angiogenesis inhibiting polypeptide using a viral vector or plasmid which can be administered so that cells of the patient in the vicinity of the target site are infected or transfected with the nucleic acid encoding the angiogenic-inhibiting polypeptide. Furthermore, like the instant application, the WO 99/26480 document teaches in detail a number of different viral vectors that can be used to deliver and express the therapeutic endostatin protein (e.g., see page 5). The WO document indicates that the term "a gene therapy vector" is meant to mean a vector useful for gene therapy and can be a virus, plasmid or phage (see page 5). The WO 99/26480 document teaches, "preferred vectors include, e.g., retroviral vectors, adenoviral vectors, adeno-associated vectors, herpes virus vectors, Similiki Forest Virus-based vectors, Human Immunodeficiency Virus, Simian Immunodeficiency virus, and non-viral plasmids" (see page 5). Additionally, page 9 of the WO 99/26480 document teaches, in detail, a preferred embodiment in constructing a gene therapy vector that is sufficient for use in the treatment of angiogenesis in vivo. WO 99/26480 also explicitly teaches that the eye is a specific target for the delivery of the therapeutic nucleic acid (e.g., see page 14, lines 1-15). Therefore, WO 99/26480 teaches each and every element of the instant claims. Therefore, the WO 99/26480 document clearly teaches using a vector that expresses endostatin for inhibiting angiogenesis in the eye of a patient

suffering from diabetic retinopathy, and discloses a number of specific vectors and methods of administration for accomplishing the treatment.

Furthermore, the Applicants have not indicated any specific critical element that the WO 99/26480 document fails to teach which prevents the document from providing an enabling disclosure. For instance, evidence that endostatin does not inhibit angiogenesis in the eye would indicate that Leboulch et al. is not enabled.

It is acknowledged that the WO 99/26480 document does not disclose a working example for the indicated method. However, in view of the state of the prior art with respect to gene therapy of the eye as well as the state of the art with respect to using endostatin as an anti-angiogenic factor in protein and gene therapy, the WO 99/26480 document does provide a sufficient disclosure to enable the indicated method.

Therefore, Applicants arguments are not persuasive and the rejections are maintained.

Conclusion

No claim is allowed.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Jon Eric Angell whose telephone number is 571-272-0756. The examiner can normally be reached on Mon-Fri, with every other Friday off.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Peter Paras can be reached on 571-272-4517. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

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PATENT EXAMINER

J.E. Angell, Ph.D.
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Intraocular expression of endostatin reduces VEGF-induced retinal vascular permeability, neovascularization, and retinal detachment

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ABSTRACT

Endostatin, a proteolytic fragment of collagen XVIII, is an endogenous inhibitor of tumor angiogenesis that also inhibits choroidal neovascularization. In this study, we assessed the effects of increased intraocular expression of endostatin on vascular endothelial growth factor (VEGF)-induced changes in the retina. After subretinal injection of a pair of gutless adenoviral vectors (AGV) designed to provide tamoxifen-inducible expression of endostatin, diffuse endostatin immunoreactivity was induced thoroughout the retina by administration of tamoxifen. Induction of endostatin in double transgenic mice with doxycycline-induced expression of VEGF in the retina resulted in significant suppression of leakage of intravascular [^{3}H]mannitol into the retina. The ability of endostatin to reduce VEGF-induced retinal vascular permeability was confirmed by using [^{3}H]mannitol leakage and two other parameters, fluorescein leakage and retinal thickness, after subretinal injection of a bovine immunodeficiency lentiviral vector coding for endostatin (BIV-vectored endostatin, or BIVendostatin). Subretinal injection of BIVendostatin resulted in more discrete, less intense staining for endostatin in the retina than that seen with the inducible AGV system, which suggested lower levels and allowed visualization of sites where endostatin was concentrated. Endostatin staining outlined retinal blood vessels, which suggested endostatin binding to a component of vessel walls. More prolonged or higher level expression of VEGF in the retina resulted in neovascularization and retinal detachment, both of which were also significantly reduced by BIVendostatin. These data suggest that endostatin may be an endogenous inhibitor of vasopermeability as well as neovascularization. In patients with diabetic retinopathy, endostatin gene transfer may provide a way to decrease the risk of three causes of visual loss: macular edema, neovascularization, and retinal detachment.

Key words: blood-retinal barrier • diabetic retinopathy • macular edema • ocular neovascularization • vascular endothelial growth factor

Intravascular injection of endostatin, the 20-kDa fragment of the NCI domain of collagen XVIII, results in regression and decreased vascularity of tumors in mice (1). Gene transfer offers an appealing way to provide sustained delivery of endostatin, and in several mouse models it has been shown that tumor growth is inhibited after endostatin gene transfer (2–5). We previously demonstrated that intravascular injection of adenoviral vectors encoding endostatin results in high serum levels of endostatin and marked suppression of choroidal neovascularization (6). Therefore, in addition to inhibiting tumor neovascularization, endostatin inhibits at least one type of ocular neovascularization.

One of the advantages of the eye with regard to gene therapy is that it is a closed compartment, and intraocular injection of a vector provides a means to achieve high, sustained transgene expression with minimal exposure to the rest of the body. The eye also provides an ideal model system to test the effects of transgenes. The rhodopsin (rho) or interphotoreceptor retinoid binding protein (IRBP) promoters, drive high-level, retina-specific expression of transgenes in the retina (7, 8). Studies of transgenic mice with retina-specific expression of vascular endothelial growth factor (VEGF) (rho/VEGF mice) have demonstrated that increased expression of VEGF is sufficient to cause retinal neovascularization (9, 10). In addition, rho/VEGF transgenics have provided a valuable model for determining the effects of drugs or genes on VEGF-induced neovascularization (11–13). By combining retina-specific promoters with the tetracycline-inducible promoter system such that the reverse tetracycyline transactivator (rtTA) is coupled to the rho or IRBP promoter, and the tetracycline response element (TRE) is coupled to VEGF, double transgenic mice with inducible expression of VEGF in the retina (rho/rtTA-TRE/VEGF and IRBP/rtTA-TRE/VEGF mice) have been generated (14). When given doxycycline at 2 mg/ml or more in drinking water for 4–5 days or longer, these mice develop severe neovascularization and retinal detachment, reminiscent of severe proliferative diabetic retinopathy. We recently found that on the third day of treatment with 2 mg/ml doxycycline there was severe leakage from retinal vessels, with little or no neovascularization developing on the following day. By decreasing the dose of doxycycline to 0.5 mg/ml, the period of retinal vascular leakage was extended to 7 days after initiation of doxycycline, and after that neovascularization without retinal detachment occurred (P. A. Campochiaro, unpublished). Therefore, we found that by varying the dose and duration of doxycycline, these mice provide a means to test the effect of agents on VEGF-induced vascular permeability, neovascularization, or severe vascular proliferation resulting in retinal detachment.

In this study, we sought to determine the effects of endostatin on VEGF-induced pathology in the retina. For most experiments, we used a bovine immunodeficiency viral vector encoding endostatin (BIVendostatin), because we previously demonstrated that after intraocular injection these BIV vectors result in long-term expression with no identifiable toxicity, which suggests that BIV vectors may be useful for ocular gene transfer in humans (15). In addition, we tested a pair of gutless adenoviral vectors (AGV) designed to provide tamoxifen-inducible expression of endostatin.

MATERIALS AND METHODS

Generation, propagation, and purification of AGV vectors

The AGV vector AGVnull lacks a transgene expression cassette and was previously described (16). The AGV vectors encoding the tamoxifen-inducible chimeric transcription factor

(AGVas521) and the regulatable endostatin transgene (AGVC7mEndo) were generated from the plasmids pAGVas521 and pAGVC7mEndo, respectively. In addition to the transgene expression cassette (see below), the AGV plasmids contain the left and right inverted terminal repeats flanked by unique *Pac*I sites, the packaging signal of Ad5, and ~25 kb of the human α-synuclein intronic region as a DNA "stuffer" (16). The plasmid pAGVas521 contains the tamoxifen-inducible chimeric transcription factor composed of the unique zinc finger DNA binding domain, a modified ligand binding domain based on the human estrogen receptor, and the transactivating region derived from VP16 driven by the CMV promoter (17). To construct pAGVas521, the chimeric transcription factor expression cassette was isolated from pAvCv-C7LBD (17) by digestion with *Nru*I and *Bam*HI, and inserted into pBLSV2. The plasmid pBLSV2 was derived from pBluescript (Strategene, La Jolla, CA) and contained two multicloning site polylinkers. The resulting plasmid pBLSV2as521 was digested with *Bsp*EI and ligated to pGTI.24aPL2 digested with *Xma*I, to generate pGTI24as521. pGTI.24aPL2 contained a multicloning site polylinker flanked by human synuclein stuffer DNA. Next, pGTI24as521 was digested with *Pac*I to liberate the plasmid backbone and was combined with *Pme*I-*Mlu*I-digested pBV2, by using homologous recombination in BJ5138 *Escherichia coli* (18), to generate pAGVas521. The plasmid pBV2 contains 26625 bp of human synuclein stuffer DNA.

The plasmid pAGVC7mEndo contains the ligand-inducible transcription factor-regulated promoter driving the expression of murine endostatin. This expression cassette was previously described (17). To construct pAGVC7mEndo, the plasmid pav-6X2C7tatamendo (17) was digested with *Asc*I (blunted) and *Bam*HI and inserted into pBLSV2C7endo. The plasmid pBLSV2C7endo was digested with *Bam*HI and *Eco*RI, with the ends filled in, and was ligated to pGTI245.aPL2 digested with *Sma*I to generate pGTI24C7endo. Finally, pGTI245.aPL2 was digested with *Pac*I to liberate the plasmid backbone and was combined with *Pme*I- and *Mlu*I-digested pBV4, by using homologous recombination in BJ5138 *E. coli* (18) to generate pAGVC7mEndo. The plasmid pBV4 contains 27,191 bp of human synuclein stuffer DNA.

Gutless vector generation and large-scale production and purification were performed as described before (16). The particle titers were determined by optical density measurements (19). DNA extracted from CsCl-purified vectors was analyzed by restriction enzyme digestions to verify vector integrity. A hexon-based quantitative polymerase chain reaction (PCR) assay was used to determine the level of helper virus contamination in AGV preparations (16). Helper contamination levels of AGVnull, AGVas521, and AGVC7mEndo preparations used in this study were 0.09%, 1.9%, and 1.4%, respectively.

This tamoxifen-inducible system displays high-level inducible endostatin expression in the serum of C57BL/6 mice after systemic administration of early generation, E1/E2a/E3-deficient vectors encoding either the inducible transcription factor or the regulatable endostatin transgene (17). When this system was incorporated into AGV vectors, extremely high level, repeatedly inducible endostatin expression was observed after administration of the vectors to mice (D. Zerby et al., 2003, Human Gene Therapy, in press).

Generation, propagation, and purification of BIVendostatin

The transfer vector BIVendostatin was derived from pBSV4MGpptGAG, a BIV-based transfer vector construct encoding enhanced green fluorescent protein (eGFP) under MND U3 promoter (15). The eGFP coding sequence was replaced with murine endostatin (mEndo). Specifically, to

delete eGFP from the parent plasmid pBSV4MGpptGAG, the eGFP plus some flanking sequence was amplified by PCR. The primers used were eGFP1FOR, 5'-GCGCATGTCGACAGAATATGGGCCAAAC-3', which incorporated an *Sal*I site to the 5'-end of the PCR product; and eGFP1REV, 5'-GCGCTACTGCAGAGCTAATGAGCTACAC-3', which incorporated a *Pst*I site to the 3'-end of the PCR product. This fragment was cut with *Sal*I and *Pst*I and ligated with pBSII(KS+), which was also previously digested with *Sal*I and *Pst*I, to create pBS2eGFP. The ExSite PCR-Based Site-Directed Mutagenesis Kit (Stratagene) was used to delete eGFP from pBS2eGFP. PCR primers were designed that flanked the outer portions of the eGFP gene and pointed outward, thus amplifying everything, including the entire flanking sequence and plasmid, except eGFP. The primers used were DELeGFP1FOR: 5'-CCGGCTAGCTTAAGGGTGGCGACCGGT-3', which added *Nhe*I and *Afl*II restriction sites; and DELeGFP1REV: 5'-GCTTCGAACGCGTAGCGGCCAACCCTC-3', which added *Bst*BI and *Mlu*I restriction sites. The amplicon was treated according to the manufacturer's instruction and was ligated to form pBS2deleGFP with eGFP deleted but with the flanking sequence from the parental plasmid remaining. This strategy also created four new restriction sites in the middle. This fragment was sequenced to ensure that no mutations had been introduced. The mEndo gene insert was prepared from a described previously adenoviral shuttle plasmid, pAVmEndolxr (6). This plasmid was digested with *Nhe*I and *Cla*I to release the mEndo fragment. This fragment was ligated with pBS2deleGFP, which was previously digested with *Nhe*I and *Bst*BI (compatible ends with *Cla*I), to generate pBS2deleGFPmEndo. The woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) was inserted downstream of mEndo at the *Mlu*I site, to create pBS2deleGFPmEndoPRE. Finally, the plasmid pBS2deleGFPmEndoPRE was digested with *Bsu*36I and *Bbv*CI to release the mEndo coding sequence and the orignal eGFP flanking sequences, and this fragment was ligated with pBS4MGpptGAG, which was previously digested with *Bsu*36I and *Bbv*CI, to generate pBvMNDmEndoPRE. A null BIV vector, pBvMNDPRE, was also generated and served as a negative control vector. pBvMNDPRE was identical to pBvMNDmEndPRE except for the absence of the mEndo coding sequence.

BIV-based lentiviral vector production via transient transfection methods was previously described (15, 20). Briefly, to generate BIV vector particles encoding mEndo (BIVendostatin), 293T cells in 150-mm dishes (2×10^7 cells/dish) were transfected with 45 μg of pBvMNDmEndoPRE, 45 μg of BIV-based packaging construct pBIVminipack, and 13.5 μg of pseudotyping envelope expression construct pVSV-G (20). To generate BIVnull vectors, pBvMNDmEndoPRE was replaced with the pBvMNDPRE construct. Forty-eight hours after transfection, the vector was harvested and filtered through a 0.45-μm filter. The vector supernatant was then concentrated by ultracentrifugation (15). The concentrated vector was separated into aliquots and stored at –80°C until used.

The concentrated vector was assayed for reverse transcriptase (RT) activity, a measure of vector particles. Both BIVendostatin and BIVnull vectors scored RT activity of about 15 μg/ml. We previously showed, with a BIV-based vector encoding eGFP, that 1 ng of RT equals about 1×10^5 transducing units (TU) (20). Therefore, the estimated titer of BIVendostatin vector was 1.5×10^9 TU/ml. To examine whether the BIVendostatin vector could mediate efficient production of mEndo, Cf2Th cells in a 6-well plate (4×10^5 cells/well) were transduced with either 1 μl (15 ng of RT equivalent vector particles) of BIVnull vector or the same amount of BIVendostatin vector. Forty-eight hours after transduction, the cell supernatant was assayed for endostatin

expression by use of a commercial endostatin assay kit, Accucyte Mouse Endostatin assay system, following the manufacturer's instruction (Cytimmune Sciences, College Park, MD). BIVendostatin vector-transduced cells produced 412 ng/ml endostatin, whereas the BIVnull vector did not score a detectable level of endostatin.

Double transgenic mice with inducible expression of VEGF in the retina

Two lines of double transgenic mice with inducible expression of VEGF in the retina have been generated and characterized (14) and were used in this study. In one of the lines, the IRBP promoter is combined with the reverse tetracycline transactivator (rtTA) system to direct doxycyline-inducible expression of VEGF in photoreceptors. These mice are referred to as IRBP/rtTA-TRE/VEGF mice. In the second line of double transgenic mice, the rho promoter rather than the IRBP promoter is combined with the rtTA system to direct doxycyline-inducible expression of VEGF in photoreceptors. These mice are referred to as rho/rtTA-TRE/VEGF mice.

Intraocular injection of vector

Mice were treated humanely in strict compliance with the Association for Research in Vision and Ophthalmology statement on the use of animals in research. Subretinal injections of vector were done as described previously (21). For studies with AGV, adult mice were given a subretinal injection of 6×10^7 particles of a 1:1 mixture of AGVC7mEndo and AGVas521 in one eye and 6×10^7 particles of AGVnull in the other eye. For studies with lentiviral vectors, the mice received 1.5×10^6 TU of BIVendostatin in one eye and 1.5×10^6 TU of BIVnull in the other eye. Pulled glass micropipets were calibrated to deliver 1 μl of vehicle on depression of a foot switch. Injections were performed using a condensing lens system on the dissecting microscope and a contact lens, which allowed visualization of the retina during the injection. The mice were anesthetized, pupils were dilated, and, under a dissecting microscope, the sharpened tip of the micropipette was passed through the sclera posterior to the limbus and was positioned just above the retina. Depression of the foot switch caused the jet of injection fluid to penetrate the retina. This technique is very atraumatic, and the direct visualization allows confirmation of a successful injection because of the appearance of a small retinal detachment (bleb). The blebs were quite uniform in size and involved slightly less than half of the retina.

Immunohistochemistry for endostatin

Eyes were punctured and placed in a solution of 4% paraformaldehyde plus 5% sucrose and were then incubated overnight in 0.1 M phosphate buffer, pH 7.4, for 1.5 h at 4°C. Eyes were then rinsed and rapidly frozen in a 2:1 mixture of 0.1 M phosphate buffer plus 20% sucrose in OCT. Frozen sections of 10 μm were dried and postfixed in cold 4% paraformaldehyde for 30 min. After rinsing, slides were blocked with cold methanol containing 6.25% H_2O_2 for 15 min and then with 2% skim milk in Tris-buffered saline (TBS) for 30 min at room temperature. Slides were incubated in 1.5 μg/ml polyclonal goat IgG directed against mouse endostatin (R&D Systems, Minneapolis, MN) in 2% milk in TBS for 1 h at room temperature. After washing in 0.1% skim milk in TBS for 10 min, slides were incubated 30 min at room temperature in 2 μg/ml biotin-conjugated anti-goat IgG (Santa Cruz Biotechnology, Santa Cruz, CA) in 2% skim milk in TBS. After washing 10 min in 0.1% milk in TBS, slides were incubated for 30 min at room temperature in streptavidin-phosphatase (Kirkegaard and Perry, Cabin John, MD). After three 5-

min washes in 0.05 M Tris-HCl, slides were developed with HistoMark Red (Kirkegaard and Perry) and mounted.

Measurement of retinal vascular permeability by using [^{3}H]mannitol as tracer

Adult double transgenic IRBP/rtTA-TRE/VEGF mice (n = 11) were given a subretinal injection of 6×10^7 particles of a 1:1 mixture of AGVC7mEndo and AGVas521 in the right eye and 6×10^7 particles of AGVnull in the left eye. The next day mice started to receive daily intraperitoneal (i.p.) injections of 500 μg of tamoxifen in 5% dimethyl sulfoxide (DMSO) in sunflower oil, and after 3 days they were given 2 mg/ml doxycycline in their drinking water. On the third day after doxycycline was initiated, retinal vascular permeability was measured by using [^{3}H]mannitol as described previously (22). Briefly, mice were given an i.p. injection of [^{3}H]mannitol at 1 μCi/g of body weight (New England Nuclear, Boston, MA) and were killed after 1 h, and eyes were removed. The cornea and lens were removed, and the entire retina was carefully dissected from the eyecup and placed within preweighed scintillation vials. The thoracic cavity was opened, and the left superior lobe of the lung was removed and placed in another preweighed scintillation vial. A left dorsal incision was made, and the retroperitoneal space was entered without entering the peritoneal cavity. The renal vessels were clamped with a forceps, and the left kidney was removed, cleaned of all fat, and placed into a preweighed scintillation vial. All liquid was removed from the vials, and remaining droplets were allowed to evaporate over 20 min. The vials were weighed and the tissue weights were recorded. One ml of NCSII solubilizing solution (Amersham, Chicago, IL) was added to each vial, and the vials were incubated overnight in a water bath at 50°C. The solubilized tissue was brought to room temperature and decolorized with 20% benzoyl peroxide in toluene in a 50°C water bath. The vials were brought to room temperature, and 5 ml of Cytoscint ES (ICN, Aurora, OH) and 30 μl of glacial acetic acid were added. The vials were stored for several hours in darkness at 4°C to eliminate chemoluminescence. Radioactivity was counted via a Wallac 1409 Liquid Scintillation Counter (Gaithersburg, MD).

In other experiments, IRBP/rtTA-TRE/VEGF mice (n = 20) were given a subretinal injection of 1.5×10^6 TU of BIVendostatin in the right eye and 1.5×10^6 TU of BIVnull in the left eye. Four weeks after vector injection, mice started to receive 2 mg/ml doxycycline in their drinking water, and on day 3 of treatment the retinal vascular permeability was measured by using [^{3}H]mannitol as tracer.

Assessment of retinal vascular permeability by using fluorescein leakage

Adult rho/rtTA-TRE/VEGF double transgenic mice (n = 6) were given subretinal injections of 1.5×10^6 TU of BIVendostatin in the right eye and 1.5×10^6 TU of BIVnull in the left eye. Four weeks after vector injection, mice started to receive 0.5 mg/ml doxycycline in their drinking water. Four or 7 days later, mice were given an i.p. injection of 1% fluorescein sodium at 12 μl/g of body weight (Alcon, Fort Worth, TX), and after 5 min the retinas were examined by in vivo fluorescence microscopy. Pictures were taken of the left eye and then within 30 s of the right eye. Seven days after initiating VEGF expression, fluorescein angiography was repeated, and mice were killed, and retinal frozen sections were cut through the posterior part of the retina adjacent to the optic nerve in the same location in each eye. The sections were stained with biotinylated *Griffonia simplicifolia* lectin B4 (GSA; Vector Laboratories, Burlingame, CA), which binds selectively to vascular cells (23). Slides were incubated in methanol/H_2O_2 for 10

min at 4°C, washed with 0.05 M TBS, pH 7.6, and incubated for 30 min in 10% normal porcine serum. Slides were incubated for 2 h at room temperature with biotinylated GSA. After the slides were rinsed with 0.05 M TBS, they were incubated with avidin coupled to peroxidase (Vector Laboratories) for 45 min at room temperature. After a 10-min wash in 0.05 M TBS, slides were incubated with diaminobenzidine (Research Genetics, Huntsville, AL), to give a brown reaction product, and were counterstained with hematoxylin and eosin.

Measurement of retinal thickness

Adult rho/rtTA-TRE/VEGF double transgenic mice (n = 8) were given subretinal injections of 1.5×10^6 TU of BIVendostatin in the right eye and 1.5×10^6 TU of BIVnull in the left eye. Four weeks after vector injection, mice were started to receive 0.5 mg/ml doxycycline in their drinking water. Seven days after doxycycline was initiated, mice were killed and 10-μm frozen sections were cut through the posterior part of the retina adjacent to the optic nerve in the same location in each eye. The sections were stained with GSA as described above. Retinal thickness was measured by image analysis 300 μm from each edge of the optic nerve, and measurements were averaged to give a single experimental value.

Assessment of retinal neovascularization in double transgenic mice

Three rho/rtTA-TRE/VEGF mice were used to assess the effect of endostatin on VEGF-induced retinal neovascularization. Four weeks after subretinal injection of 1.5×10^6 TU of BIVendostatin in the right eye and 1.5×10^6 TU of BIVnull in the left eye, mice started to receive 0.5 mg/ml doxycycline in their drinking water. Seven days after doxycycline was initiated, mice were killed and each eye was sectioned (10-μm sections) from the peripheral edge of the iris to the other peripheral edge 180° away. Sections were stained with GSA, hematoxylin, and eosin. Three mice were killed 10 days after initiation of doxycycline. Serial sections were cut through the entire eyes and stained with GSA, hematoxylin, and eosin. The area of GSA staining in the photoreceptor layer is an indication of the amount of neovascularization and was measured on sections 100 μm apart from one edge of the iris to the other edge (usually 13 sections per eye). The average of these 13 measurements constituted a single experimental value, the area of neovascularization per section.

Assessment of retinal detachment in double transgenic mice

Eleven adult rho/rtTA-TRE/VEGF double transgenic mice were given subretinal injections of 1.5×10^6 TU of BIVendostatin in the right eye and 1.5×10^6 TU of BIVnull in the left eye. Four weeks after vector injection, mice started to receive 2 mg/ml doxycycline in their drinking water. On the fifth day after initiation of doxycycline, mice were anesthetized, pupils were dilated, and an ophthalmoscopic examination was performed on each eye by two independent observers who noted whether there was a total or a partial retinal detachment, or no detachment.

Statistical comparisons

Experiments were designed so that there were fellow-eye controls and therefore most comparisons were done via a paired t test. However, there were three possible outcomes in the assessment for retinal detachment, and the distribution of outcomes was therefore compared between BIVendostatin-injected vs. BIVnull-injected eyes, with an exact test for marginal

homogeneity for ordered categories (24, 25). The analysis was performed with StatXact software (Cytel Software, Cambridge, MA).

RESULTS

Tamoxifen-induced expression of endostatin in the retina with the AGV system

Subretinal injection of AGV encoding *β-galactosidase* causes very strong transduction of retinal pigmented epithelial cells and sporatic transduction of overlying retinal cells. Transgene expression decreases somewhat over time but is still substantial 5 months after vector injection. We explored AGV gene transfer of an estrogen receptor-based inducible expression system (17) to assess its feasibility for inducible expression of endostatin in the retina. Adult C57BL/6 mice were given a subretinal injection of 6×10^7 particles of AGVnull or 6×10^7 particles of a 1:1 mixture of AGVC7mEndo and AGVas521. After 7 days, mice injected with AGVnull and half of the mice injected with the vector pair started to receive tamoxifen; the other half of the mice injected with the vector pair were treated with vehicle. Fourteen days after initiation of tamoxifen, mice injected with AGVnull that were treated with tamoxifen (Fig. 1A) and mice injected with the vector pair constituting the inducible system treated with vehicle (Fig. 1B) showed staining only along the internal limiting membrane and the Bruch membrane, which is likely to be due to cross-reactivity with collagen XVIII, a known component of these membranes (26). Mice injected with the vector pair constituting the inducible system treated with tamoxifen showed prominent staining for endostatin throughout the entire retina (Fig. 1C), which demonstrated strong induction of endostatin expression in the retina.

Tamoxifen-induced expression of endostatin suppressed VEGF-induced retinal vascular permeability

Adult IRBP/rtTA-TRE/VEGF mice were given a subretinal injection of 6×10^7 particles of a 1:1 mixture of AGVC7mEndo and AGVas521 in the right eye and 6×10^7 particles of AGVnull in the left eye. After treatment with tamoxifen for 4 days to induce endostatin expression, mice were given 2 mg/ml doxycycline in drinking water to coexpress VEGF. On the third day after initiation of doxycycline, retinal vascular permeability was measured by using [^{3}H]mannitol as tracer. Both the retina-to-lung (RLLR) and retina-to-renal (RRLR) leakage ratios were significantly reduced in eyes with induced expression of both endostatin and VEGF compared with fellow eyes in which there was expression of VEGF alone (Fig. 1D). This demonstrates that endostatin suppressed the increased permeability of retinal vessels that usually occurs in the presence of VEGF.

Expression of endostatin in the retina and RPE cells 4 wk after subretinal injection of BIVendostatin

We previously demonstrated that subretinal injection of a lentiviral vector derived from BIV promotes long-term transgene expression in RPE cells with no identifiable toxicity (15). We sought to determine whether BIVendostatin also had a vascular-stabilizing effect in the retina. Adult C57BL/6 mice were given subretinal injections of 1.5×10^6 TU of BIVendostatin in the right eye and 1.5×10^6 TU of BIVnull in the left eye. Four weeks after vector injection, mice were killed and ocular frozen sections were immunohistochemically stained for endostatin using HistoMark Red, which provides a red reaction product. Eyes injected with BIVendostatin (Fig.

2A and B) showed heavy staining for endostatin in RPE cells (Fig. 2B, large arrows) and throughout the inner nuclear layer, with dense staining of the walls of some blood vessels (Fig. 2B, small arrows). The stained linear structures in the inner plexiform layer (Fig. 2B, arrowhead) are typical of Müller cell processes. Eyes injected with BIVnull showed reaction product along the internal limiting membrane (Fig. 2C, arrowheads) and the Bruch membrane (Fig. 2C, arrows), which, as noted above, is likely to be due to cross-reactivity with collagen XVIII (26).

BIVendostatin suppressed VEGF-induced retinal vascular permeability

Adult double transgenic IRBP/rtTA-TRE/VEGF mice were given a subretinal injection of 1.5×10^6 TU of BIVendostatin in the right eye and 1.5×10^6 TU of BIVnull in the left eye. Four weeks after vector injection, mice started to receive 2 mg/ml doxycycline in their drinking water, and on the third day, retinal vascular permeability was measured via [^{3}H]mannitol. RLLR was significantly reduced in eyes expressing endostatin compared with fellow eyes that had been injected with BIVnull (Fig. 2D). Therefore, expression of endostatin with either of two different vector systems blocks VEGF-induced increased vascular permeability in the retina.

Increased expression of endostatin suppressed VEGF-induced fluorescein leakage and retinal thickening

Retinal vascular permeability is assessed clinically in two ways: fluorescein angiography and measurement of retinal thickness. Fluorescein angiography is performed by intravascular injection of sodium fluorescein dye followed by retinal photography with an excitation filter that blocks all but peak absorption wavelengths for fluorescein and a barrier filter that blocks all but peak emission wavelengths. This technique allows direct visualization and thereby localization of leakage, but it does not lend itself well to quantitation of leakage. We have adapted the technique to mice by injecting sodium fluorescein i.p. and performing in vivo fluorescence microscopy.

Adult rho/rtTA-TRE/VEGF mice were given subretinal injections of 1.5×10^6 TU of BIVendostatin in the right eye and 1.5×10^6 TU of BIVnull in the left eye. Four weeks after injection, mice started to receive 0.5 mg/ml doxycycline in their drinking water. Fluorescein angiography 4 days after initiation of doxycycline showed normal-appearing retinal vessels with distinct walls, which indicated little or no permeation of fluorescein through retinal vessels in BIVendostatin-injected eyes (Fig. 3A). In contrast, BIVnull-injected eyes showed blurred vessel walls and diffuse fluorescence throughout the retina, which indicated extensive extravasation of fluorescein from retinal vessels (Fig. 3B). Seven days after initiation of doxycycline, there was still little evidence of fluorescein leakage in BIVendostatin-injected eyes (Fig. 3C), compared with massive leakage in BIVnull-injected eyes (Fig. 3D).

When retinal vascular permeability is increased, fluid collects in the retina, which results in retinal thickening. Thickening in the macula is called macular edema, and there is an inverse correlation between the amount of abnormal thickening and visual acuity. Thus, retinal thickening is a physiologically relevant measurement and lends itself to precise quantitation via in vivo imaging techniques such as optical coherence tomography (OCT) or retinal thickness analysis (27). Seven days after administration of 0.5 mg/ml doxycycline was started in drinking water to induce VEGF expression, mice were killed. Frozen sections from the posterior portion

of BIVendostatin-injected eyes (Fig. 3E) showed less retinal thickening than did sections from a corresponding region in BIVnull-injected eyes (Fig. 3F).

Eight rho/rtTA-TRE/VEGF mice were used to quantitatively assess the effect of endostatin on VEGF-induced thickening of the retina. Four weeks after subretinal injection of 1.5×10^6 TU of BIVendostatin in the right eye and 1.5×10^6 TU of BIVnull in the left eye, mice started to receive 0.5 mg/ml doxycycline in their drinking water. Seven days after doxycycline was started, retinal thickness was measured by image analysis 300 μm from each edge of the optic nerve along the horizontal meridian and averaged to give a single experimental value in each eye. Mean retinal thickness was significantly greater in BIVnull-injected eyes (Fig. 4A) than in BIVendostatin-injected eyes (Fig. 4B and E).

Increased expression of endostatin reduced VEGF-induced neovascularization and retinal detachment

Four weeks after subretinal injection of 1.5×10^6 TU of BIVendostatin in the right eye and 1.5×10^6 TU of BIVnull in the left eye, mice started to receive 0.5 mg/ml doxycycline in their drinking water. Ten days after doxycycline was started, the amount of retinal neovascularization was visibly greater in BIVnull-injected eyes (Fig. 4C) compared with BIVendostatin-injected eyes (Fig. 4D and F).

When rho/rtTA-TRE/VEGF mice are given 2 mg/ml doxycycline in their drinking water for 4 days or longer, they express high levels of VEGF and develop severe neovascularization and retinal detachment (14). Eleven rho/rtTA-TRE/VEGF mice were used to assess the effect of BIVendostatin on this severe phenotype resulting from prolonged high levels of retinal VEGF. Four weeks after subretinal injection of 1.5×10^6 TU of BIVendostatin in one eye and 1.5×10^6 TU of BIVnull in the other eye, mice started to receive 2 mg/ml doxycycline in their drinking water. Four days after doxycycline administration was started, two observers, masked with respect to vector group, performed an ophthalmoscopic examination and graded each eye as no, partial, or total retinal detachment. There was complete agreement between the two examiners. The percentages of eyes with no, partial, or total retinal detachment were 36.4%, 45.5%, and 18.2%, respectively, in BIVendostatin-injected eyes; the corresponding values for BIVnull-injected eyes were 9.1%, 9.1%, and 81.8% (Fig. 5). These distributions are significantly different ($P = 0.0078$) on the basis of an exact test for marginal homogeneity for ordered categories. Specifically, BIVendostatin-injected eyes were more likely to have no or partial detachment compared with BIVnull-injected eyes, which were more likely to have total detachment.

DISCUSSION

In this study, we demonstrated that increased expression of endostatin in the retina reduces VEGF-induced retinal vascular permeability. Two different vector systems were used to express endostatin and the same vascular-stabilizing effect resulted, which indicated that the effect was independent of the gene delivery vehicle and completely attributable to endostatin. This ability of endostatin to counter the leakage-promoting activity of VEGF was demonstrated with a quantitative radiolabeled tracer technique and with fluorescein angiography, a visually demonstrative technique that is used for clinical assessment of retinal vascular leakage. Additional support was provided by demonstration that production of VEGF in the retina resulted in thickening of the retina, which was prevented by endostatin. Thickening of the retina

is due to retinal edema, which results from severe and/or prolonged leakage that overwhelms pumps and other mechanisms of fluid resorption. When edema occurs in the macula, it results in decreased vision, and therefore retinal thickness is a clinically important parameter of retinal vascular leakage. In fact, measurement of macular thickness by OCT is currently the best way to quantitatively assess the effect of new treatments for retinal vascular leakage in clinical trials. Therefore, evaluation of three different parameters that provide information about the "leakiness" of retinal vessels provided a consistent picture that strongly suggests that endostatin attenuates the ability of VEGF to cause leakage from retinal blood vessels.

The demonstration that endostatin counteracted the vasopermeability effects of VEGF is novel and potentially important. It is now widely accepted that neovascularization is controlled by a balance between angiogenic and antiangiogenic factors and that endostatin is one of many endogenous antiangiogenic factors (1, 28–32). This study suggests that vasopermeability may be modulated in a similar fashion, by a balance between pro- and antipermeability factors, and that endostatin is an endogenous antipermeability factor. A theoretical advantage of such a system is that it would provide more flexibility for physiological modulation of vascular permeability. A practical advantage of the system is that it provides a new therapeutic option, endostatin gene transfer, for treatment of macular edema. This has important clinical implications, because macular edema is the most common cause of moderate vision loss in diabetic patients (33), and VEGF is an important mediator (34).

A more expected finding was the demonstration that endostatin inhibits VEGF-stimulated retinal neovascularization. As noted above, endostatin was first identified because of its ability to inhibit tumor angiogenesis (1), and studies have suggested that endostatin inhibits other types of neovascularization including choroidal neovascularization (6, 35), for which VEGF is a critical stimulus (36). However, the ability of endostatin to reduce retinal detachment caused by prolong production of high levels of VEGF in the retina is a pleasant unanticipated finding, because even potent small molecule inhibitors of VEGF have not fared well in this extremely rigorous test of efficacy (P. A. Campochiaro, unpublished). Therefore, endostatin may provide benefit for multiple features of diabetic retinopathy, including macular edema and neovascularization, leading to retinal detachment.

The mechanism by which endostatin exerts its effects is unknown, but several possibilities are emerging. Three types of interactions have been suggested by which endostatin may alter the behavior of endothelial cells. It may interact with specific endostatin receptors located on endothelial cells, which would result in activation of intracellular signaling pathways and altered gene expression. Second, endostatin may bind to other cell surface receptors and thereby modulate their signaling. Third, endostatin may bind to extracellular proteins and alter their ability to influence endothelial cell behavior.

Low- and high-affinity endostatin binding sites are present on endothelial cells, but so far only the low-affinity receptors, glypicans, have been identified (37). Some of the molecules implicated in intracellular signaling are the Shb adaptor (38), c-*myc* (39), phosphorylated retinoblastoma gene product (40), cyclin D1 (40), and tropomyosin (41). One consequence of endostatin-induced altered intracellular signaling is enhanced apoptosis of endothelial cells participating in neovascularization, but not quiescent endothelial cells (38, 42). As for other cell surface receptors, endostatin binds to integrin αv and integrin $\alpha 5$, which may perturb cell migration and promote apoptosis (43). Endostatin also binds to VEGF receptors 1 and 2 and

thereby suppresses VEGF stimulation of endothelial cells (44). This may be particularly relevant to our findings, because it provides a potential mechanism by which expression of endostatin in the retina could suppress VEGF-induced vascular permeability, neovascularization, and retinal detachment. However, endostatin may have multiple actions that play a role, because it also perturbs matrix metalloproteinase-2 (45) and endothelial nitric oxide synthase (46), which have been implicated in ocular angiogenesis (12, 47, 48). Endostatin also blocks the interaction of endothelial cells and other cells with collagen XVIII, the basement membrane protein from which endostatin is generated by proteolytic cleavage (49, 50). Recent studies have demonstrated that collagen XVIII plays a fundamental and phylogenetically conserved role in cell migration (49, 50) and that deficiency of collagen XVIII results in delayed regression of hyaloidal blood vessels and abnormal or absent retinal vascular development (26). This phenotype is very similar to that resulting from lack of angiopoietin 2 (51, 52), which suggests that both collagen XVIII/endostatin and angiopoietin 2 are necessary for blood vessel development and vascular remodeling in the eye. Whether angiopoietin 2 and collagen XVIII/endostatin collaborate to modulate vessel growth and regression in the eye is an interesting question that deserves future investigation.

Our data suggest that endostatin not only plays a role in vascular remodeling but also influences vascular permeability. As noted above, this may simply be due to antagonism of VEGF signaling, but the effect of endostatin on cell adhesion and the cytoskeleton may also play a role. This suggestion is supported by the observation that endostatin suppresses fibroblast growth factor 2-induced loosening of cell-cell adhesions (53), and thus it may act to stabilize endothelial cell junctions and reduce permeability regardless of the nature of the vasopermeability stimuli involved.

Whether other antiangiogenic factors also have antipermeability activity is an important question. Our preliminary unpublished results indicate that pigment epithelium-derived factor, which inhibits and causes regression of ocular neovascularization (21, 54, 55), does not block VEGF-induced retinal vascular leakage. In future studies, we will investigate other antiangiogenic agents for their effect on vascular leakage in the retina. We will also examine the effects of long-term expression of endostatin in the eye, because if it does not have deleterious effects, it may be an ideal agent for treatment of diabetic retinopathy and age-related macular degeneration, both of which cause loss of visual acuity from a combination of vascular leakage and progressive neovascularization and scarring.

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Fig. 1

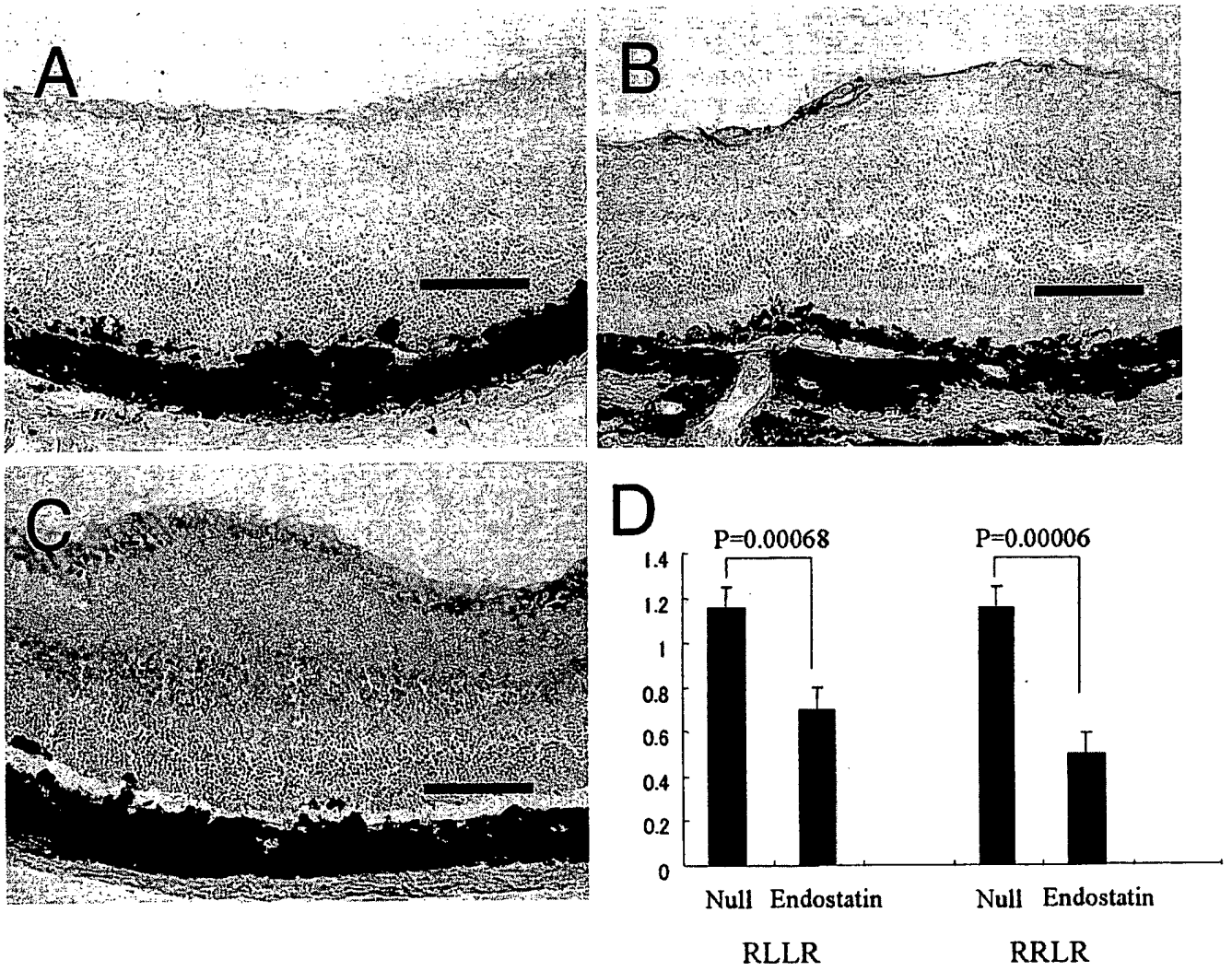

Figure 1. Tamoxifen-induced expression of endostatin in the retina with a gutless adenoviral system suppressed VEGF-stimulated vascular leakage. Adult C57BL/6 mice were given a subretinal injection of 6×10^7 particles of AGVnull (**A**) or 6×10^7 particles of a 1:1 mixture of AGVC7mEndo and AGVas521 (**B** and **C**). Seven days after injection, mice started to receive daily i.p. injections of 500 μg of tamoxifen in 5% DMSO in sunflower oil (**A** and **C**) or vehicle (**B**). Fourteen days after initiation of tamoxifen, mice were killed and ocular frozen sections were immunohistochemically stained for endostatin, as described in Materials and Methods, by using HistoMark Red, which provides a red reaction product. Mice injected with AGVnull that were treated with tamoxifen (**A**) and mice injected with the vector pair constituting the inducible system treated with vehicle (**B**) showed staining only along the internal limiting membrane (arrowheads) and the Bruch membrane (arrows), which is likely to be due to cross-reactivity with collagen XVIII, a known component of these membranes. Mice injected with the vector pair constituting the inducible system treated with tamoxifen showed prominent staining for endostatin throughout the entire retina (**C**). **D)** Adult double transgenic IRBP/rtTA-TRE/VEGF mice ($n = 11$) were given a subretinal injection of 6×10^7 particles of a 1:1 mixture of AGVC7mEndo and AGVas521 in the right eye and 6×10^7 particles of AGVnull in the left eye. The next day, mice started to receive daily i.p. injections of 500 μg of tamoxifen in 5% DMSO in sunflower oil; after 3 days they were given 2 mg/ml doxycycline in their drinking water. On day 3 of treatment with both tamoxifen and doxycycline, retinal vascular permeability was measured by using [^{3}H]mannitol as tracer, as described in Materials and Methods. Both RLLR and RRLR were significantly reduced in eyes with induced expression of endostatin compared with fellow eyes that had been injected with null vector. Statistical comparisons were made with a paired t test. Scale bars = 100 μm.

Fig. 2

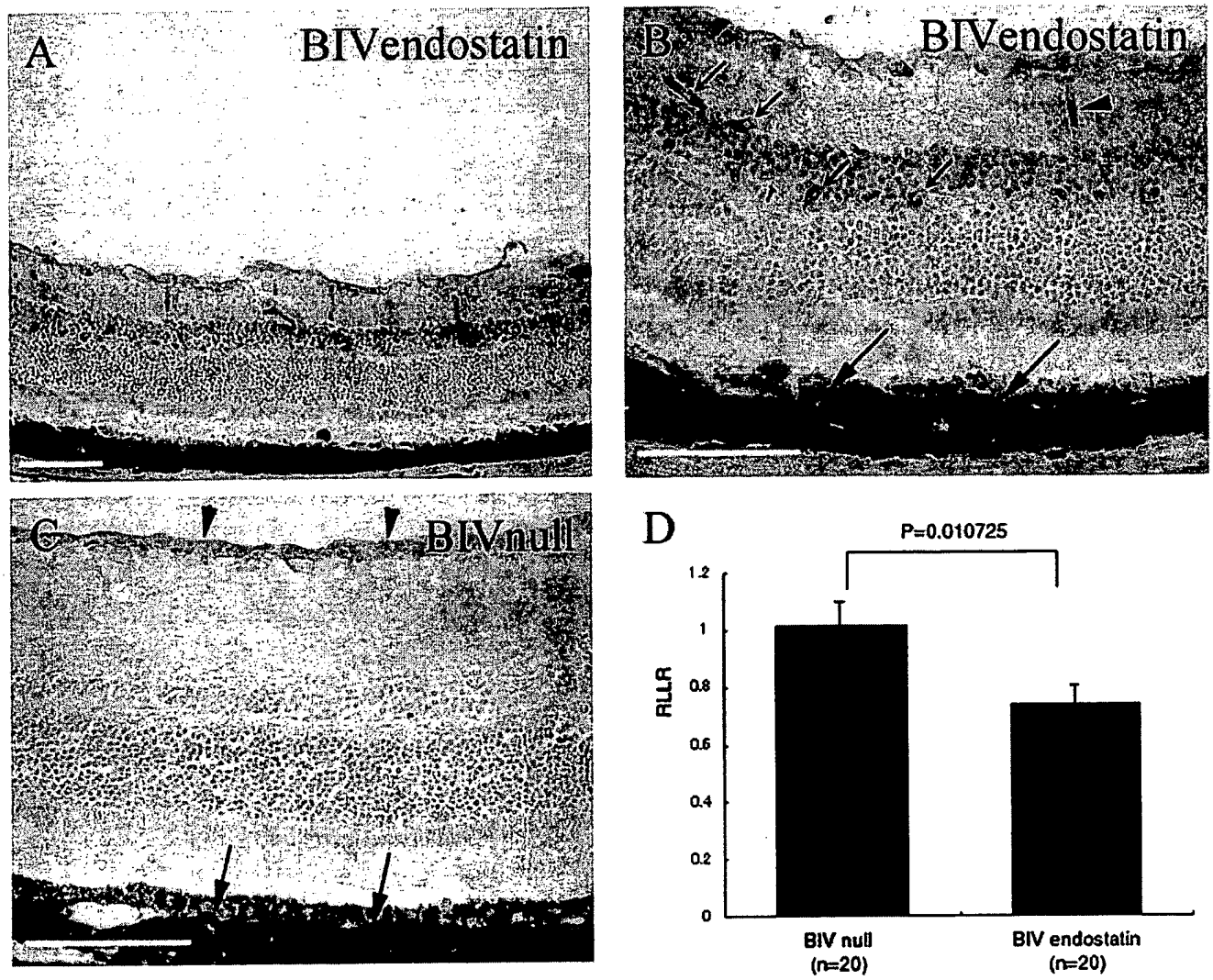

Figure 2. Expression of endostatin in the retina 4 weeks after subretinal injection of BIVendostatin reduced VEGF-stimulated vascular permeability. Adult C57BL/6 mice were given subretinal injections of 1.5×10^6 TU of BIVendostatin in the right eye and 1.5×10^6 TU of BIVnull in the left eye. Four weeks after vector injection, mice were killed and ocular frozen sections were immunohistochemically stained for endostatin, as described in Materials and Methods, by using HistoMark Red, which provides a red reaction product. Eyes injected with BIVendostatin (**A** and **B**) showed heavy staining for endostatin in RPE cells (**B**, large arrows) and throughout the inner nuclear layer with dense staining of the walls of some blood vessels (**B**, small arrows). The stained linear structures in the inner plexiform layer (**B**, arrowhead) are typical of Müller cell processes. Eyes injected with BIVnull showed reaction product along the internal limiting membrane (**C**, arrowheads) and the Bruch membrane (arrows), which is likely to be due to cross-reactivity with collagen XVIII, a known component of these membranes. **D)** Adult double transgenic IRBP/rtTA-TRE/VEGF mice ($n = 20$) were given a subretinal injection of 1.5×10^6 TU of BIVendostatin in the right eye and 1.5×10^6 TU of BIVnull in the left eye. Four weeks after vector injection, mice began to receive 2 mg/ml doxycycline in their drinking water, and on day 3 of treatment the retinal vascular permeability was measured via [^{3}H]mannitol as tracer. RLLR was significantly reduced in eyes expressing endostatin compared with fellow eyes that had been injected with null vector. Statistical comparisons were made with a paired *t* test. Scale bars = 100 μm.

Fig. 3

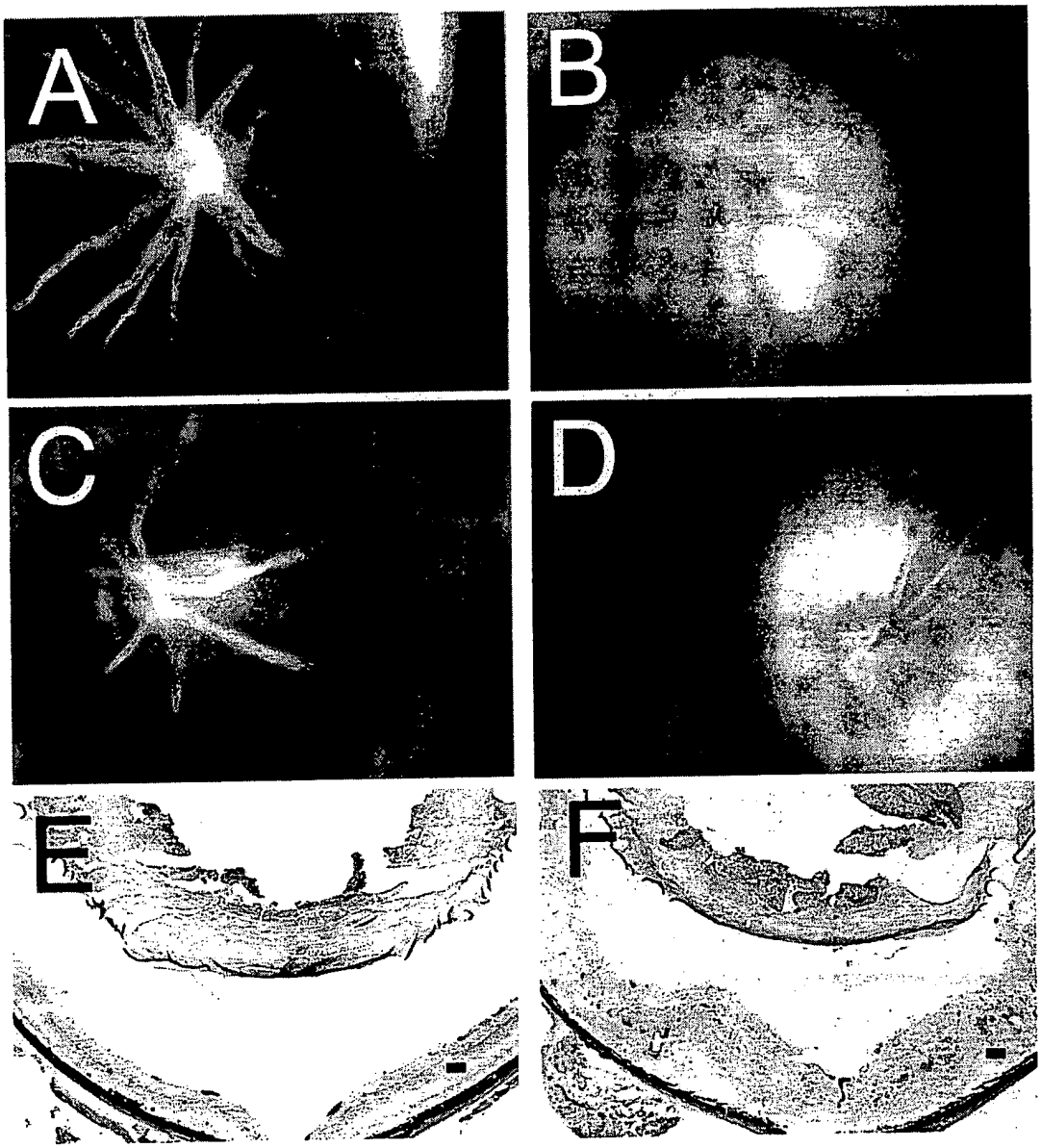

Figure 3. Intraocular BIVendostatin reduced VEGF-induced fluorescein leakage and retinal thickening. Adult rho/rtTA-TRE/VEGF double transgenic mice were given subretinal injections of 1.5×10^6 TU of BIVendostatin in the right eye and 1.5×10^6 TU of BIVnull in the left eye. Four weeks after vector injection, mice began to receive 0.5 mg/ml doxycycline in their drinking water. Four (**A** and **B**) or 7 (**C** and **D**) days later, mice were given an i.p. injection of 1% fluorescein sodium at 12 μl/g of body weight. After 5 min, retinas were examined by in vivo fluorescence microscopy and pictures were taken of the left eye and then, within 30 s, of the right eye. Four days after VEGF expression was initiated in the retina with doxycycline, the BIVendostatin-injected eye (**A**) showed much less fluorescein leakage than did the BIVnull-injected eye (**B**) of the same mouse. In another mouse, 7 days after VEGF expression was initiated in the retina with doxycycline, the BIVendostatin-injected eye (**C**) showed much less fluorescein leakage than did the BIVnull-injected eye (**D**). Seven days after VEGF expression was initiated, mice were killed and retinal frozen sections were cut through the posterior part of the retina adjacent to the optic nerve in the same location in each eye. The sections were stained with GSA, hematoxylin, and eosin. The retina in the BIVnull injected eye (**F**) was much thicker than the retina in the BIVendostatin-injected eye (**E**).

Fig. 4

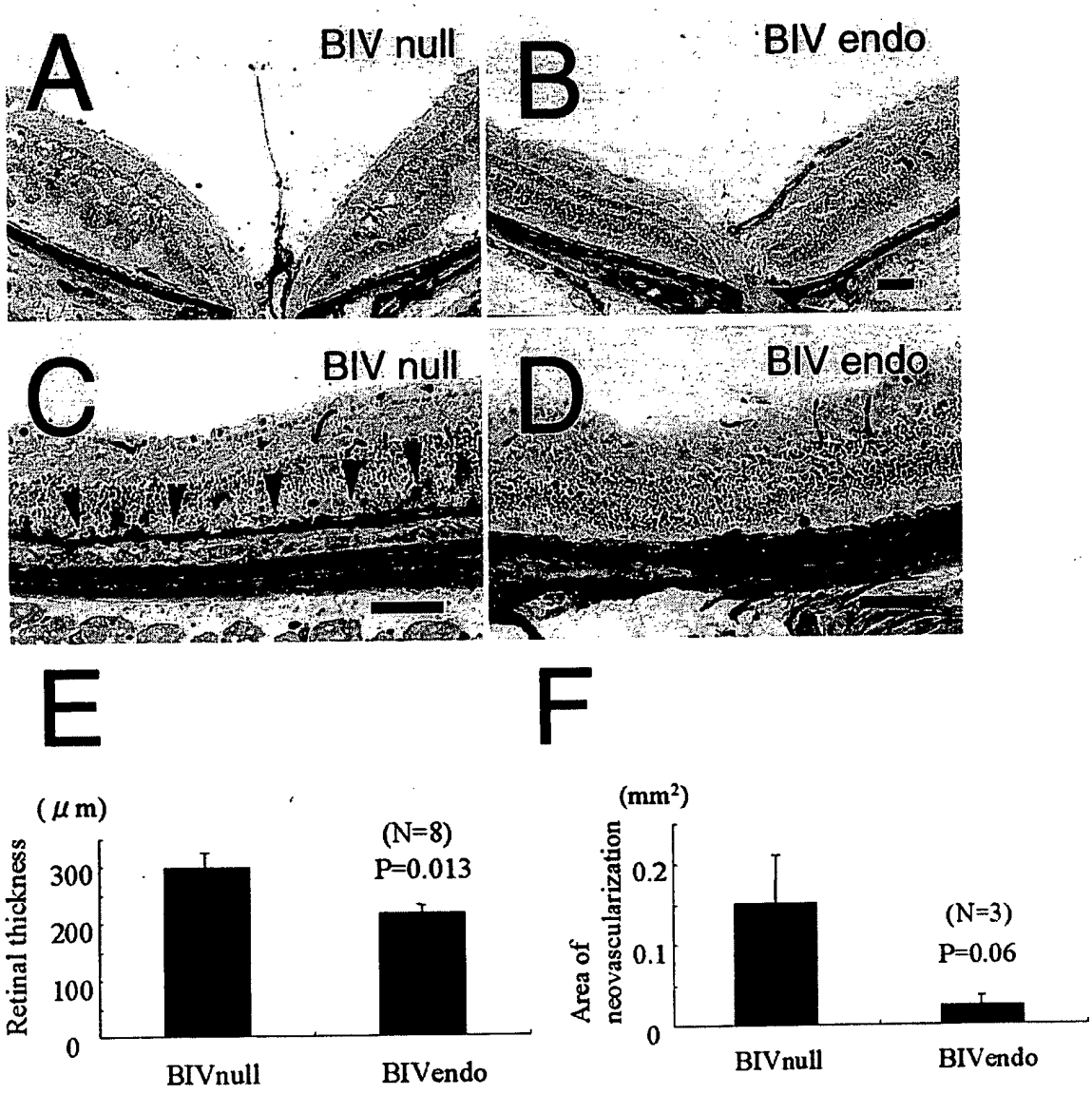

Figure 4. Intraocular BIVendostatin reduced VEGF-induced thickening and neovascularization in the retina. Adult rho/rtTA-TRE/VEGF double transgenic mice were given subretinal injections of 1.5×10^6 TU of BIVendostatin in the right eye and 1.5×10^6 TU of BIVnull in the left eye. Four weeks after vector injection, mice began to receive 0.5 mg/ml doxycycline in their drinking water. Seven days after doxycycline was started, the mice were killed and the eyes were rapidly removed and frozen. Eight mice were used to measure retinal thickness; sections were obtained through the optic nerve. Three mice were used to measure neovascularization; each eye was sectioned (10-μm sections) from the peripheral edge of the iris to the other peripheral edge 180° away. Sections were stained with GSA, hematoxylin, and eosin. Retinal thickness appeared greater in eyes injected with BIVnull (**A**) compared with eyes injected with BIVendostatin (**B**). Thickness was measured by image analysis 300 μm from each edge of the optic nerve, and measurements were averaged to give a single experimental value. Mean retinal thickness was significantly less in BIVendostatin-injected eyes than in BIVnull-injected eyes (**E**). The area of GSA staining in the photoreceptor layer is an indication of the amount of neovascularization and was measured on sections 100 μm apart from one edge of the iris to the other edge (usually 13 sections per eye). The average of these 13 measurements constituted a single experimental value, the area of neovascularization per section. The mean area of neovascularization per section was greater in BIVnull-injected eyes (**C**, arrowheads) compared with BIVendostatin-injected eyes (**D**, **F**). Statistical comparisons were made with a paired *t* test. Scale bars = 100 μm.

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